# A Cascade of Genes Related to Waardenburg Syndrome

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**On some occasions, mutations of a gene cause different syndromes that may have similar phenotypes. For example, mutations of the *MITF* gene cause Waardenburg syndrome type 2 (Tassabehji *et al*, 1994; Nobukuni *et al*, 1996) as well as Tietz syndrome (Smith *et al*, 1997). On other occasions, mutations of different genes cause an identical syndrome. Molecular analyses of these genes may provide a good opportunity to not only understand such syndromes themselves but also the biologic aspects of cells relevant to these syndromes. By analyzing the genes for Waardenburg syndrome, we showed that *PAX3*, the gene responsible for Waardenburg syndrome type 1, regulates *MITF*, the gene responsible for Waardenburg syndrome type 2. Such epistatic relationships have been shown between other genes related to Waardenburg syndrome, and likely to construct a cascade. This paper proposes such a cascade, one that involves genes for PAX3, MITF, human MyoD, MYF5, c-MET, c-KIT, tyrosinase, TRP-1, human QNR-71, SOX10, EDNRB, and EDN3.** *Key words: EDNRB/MITF/PAX3/SOX10. Journal of Investigative Dermatology Symposium Proceedings 4:126–129, 1999*

We have previously described transgenic mice, named *VGA-9*, with an insertional mutation (Tachibana *et al*, 1992). Phenotypes of homozygotes of these mice, i.e., white coat color, microphthalmia, and hearing impairment, are reminiscent of Waardenburg syndrome (WS), whose major symptoms are leucodermia, white forelock, heterochromia irides, and hearing impairment. Skin, hair follicle, eye, and cochlea of mammals contain melanocyetes. Thus, the major symptoms of WS individuals and *VGA-9* mice may be due to lack of melanocytes in these tissues. We examined *VGA-9* with special interest in their melanocyte pathology. Indeed, morphologic analysis revealed that homozygous *VGA-9* are lacking in melanocytes in affected tissues, i.e., skin, eye, and cochlea. Cochleae of mammals contain melanocytes as intermediate cells of the stria vascularis, which play an important role for production/maintenance of endolymph and thus for hearing function. Lack of strial intermediate cells caused degeneration of not only stria vascularis but also, successively, the organ of Corti that contains mechanoreceptor hair cells. Thus, all of the revealed phenotypes of *VGA-9* and WS2 can be explained by a lack of melanocytes.

These observations are consistent with the notion that the genes responsible for *VGA-9* and WS2 are involved in melanocyte differentiation/survival. Intriguingly, the murine *microphthalmia* (*mi*) gene, which is mutated in *VGA-9* and in a series of classical "microphtahlmia" mice (Hodgkinson *et al*, 1993; Steingrímsson *et al*, 1994), and its human homolog *MITF* (*microphthalmia-associated transcription factor*) gene, which is assigned to chromosome 3p14.1-p12.3 (Tachibana *et al*, 1994) and is mutated in WS2 (Tassabehji *et al*, 1994; Nobukuni *et al*, 1996), encode a protein with basic-helix-loop-helix-leucine-zipper structure. Proteins with such a structure are often transcription factors that are involved in cell differentiation. To test the hypothesis that *MITF* is involved in melanocyte differentiation, we transfected NIH-3T3 fibroblasts with a *MITF* cDNA inserted in an expression vector (Tachibana *et al*, 1996; Tachibana, 1997), and the transfection converted NIH-3T3 cells into cells with melanocytic characteristics. These cells were dendritic as melanocytes and expressed tyrosinase, tyrosinase-related protein 1 (TRP-1), and TRP-2, enzymes that catalyze melanin synthesis, although these cells did not synthesize melanin probably due to mutations of the tyrosinase gene in NIH-3T3 cells.

Besides *MITF*, four genes have been shown to be responsible for WS. *PAX3*, the first identified gene for WS, is a human homolog of mouse *Pax-3*, whose mutations were found in *splotch* mice. Mutations of *PAX3* were originally found in WS1 families, whose symptoms include distopia canthorum (lateral displacement of inner canthi of eyes) in addition to the previously mentioned major WS symptoms. Next, *PAX3* mutations were found in families of WS3, whose symptoms include deformity of upper limbs besides the major symptoms. Because distopia canthorum and upper limb deformity cannot be explained by melanocytes anomaly, and because *PAX3* encodes a transcription factor with paired domain and homeodomain, it is likely that PAX3 is involved in differentiation of cells other than melanocytes. On the other hand, it has not been explained how *PAX3* mutations cause symptoms resulting from melanocyte anomaly in WS1. Two possibilities were considered: the first is that PAX3 directly differentiates melanocytes; the second is that PAX3 differentiates melanocytes through transactivation of *MITF*. Expression of *PAX3*/*Pax-3* in melanocytes is consistent with both of these possibilities; however, we preferred the second possibility because *Pax-3* is expressed earlier and more widely than *mi* in neural crest. This paper describes how we proved our hypothesis that PAX3 transactivates the *MITF* gene, and failure of the transactivation due to *PAX-3* mutations causes WS1 symptoms related to melanocytes anomaly. Other epistatic relationships of WS genes and protein–protein interaction between their gene products is also discussed.

## MATERIALS AND METHODS

**Cloning of the MITF promoter** The approximately 2.3 kb promoter region of *MITF* was isolated from a P1 clone containing the first exon of the *MITF*

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Abbreviations: EMSA, electrophoretic mobility shift assay; *mi*, *microphthalmia*; *MITF*, *microphthalmia-associated transcription factor*; TRP-1, tyrosinase-related protein 1; WS, Waardenburg syndrome.

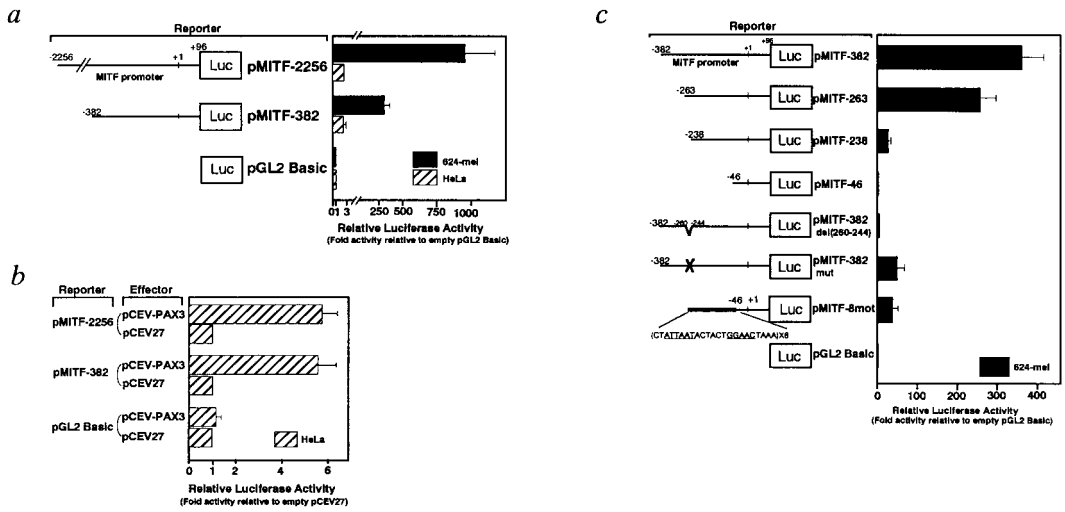


**Figure 1. PAX3 transactivates the *MITF* promoter.** The numbers shown at each reporter constructs denote the boundaries upstream and downstream of the initiation site (+1). (*a*) The *MITF* promoter is efficiently activated in melanoma cells (624-mel) but not in HeLa cells. (*b*) Co-transfected PAX3 cDNA transactivates the promoter constructs in HeLa cells. (*c*) The region between position –263 and –238 of the *MITF* promoter is critical for the promoter activity. Deletion and mutation of this region results in loss of promoter activity. Eight copies of this region fused to the basal MITF promoter is also activated. (Reproduced from Watanabe *et al*, 1998 with permission.)

gene. This region was cloned into the luciferase reporter plasmid pGL2-Basic and termed pMITF-2256. Deletion constructs pMITF-382, pMITF-263, pMITF-238, pMITF-382del(260–245), and pMITF-382mut were generated by restriction enzymes or polymerase chain reaction (PCR)-based method.

**Cloning of *PAX3* cDNA** Human *PAX3* cDNA containing a Kozak sequence and an entire coding region was generated by reverse transcriptase-polymerase chain reaction (RT-PCR) using RNA from melanoma cells. Three mutations of *PAX3* cDNA were designed from reported WS1 mutations and generated by site-directed mutagenesis. The wild-type or mutant *PAX3* cDNA were cloned into an expression vector pCEV27.

**Reporter assay** Approximately 60% confluent cells seeded 1 d before in six-well plates were cotransfected using LipofectAmine (Gibco BRL) reagents as the reporter plasmid (0.5 μg), effector plasmid (0.5 μg), and β-galactosidase expression vector PCH110 (0.2 μg, Pharmacia). After 24 h, cells were lyzed and assayed for luciferase activity.

**Electrophoretic mobility shift assay (EMSA)** Wild-type and mutated PAX3 proteins were derived from *in vitro* transcription/translation reactions. Equivalent amounts of proteins, as determined by [$^{35}$S]-methionine labeling, were incubated with end-labeled oligonucleotide of the *MITF* promoter sequence from nucleotide –265 to –236 (approximately $4 \times 10^4$ cpm). After incubation the reaction mixture was loaded on a polyacrylamice gel and then run.

Details of these procedures were fully described in the original paper (Watanabe *et al*, 1998).

## RESULTS AND DISCUSSION

Approximately 2.3 kb of the *MITF* 5′-upstream region (nucleotides–2256 to +96; transcription site 1) was cloned from a P1 library (GenBank accession number: BankIt: 153821; AF034755). This region and a shorter region (nucleotides –382 to +96) fused to the luciferase gene were activated strongly when transfected in human melanoma cells but not in HeLa cells, as judged by luciferase activity (**Fig 1***a*). We thought the difference was due to the fact that PAX3 is expressed in melanoma cells but not in HeLa cells. Consistent with this notion, ectopic expression of *PAX3* in HeLa cells transactivated the *MITF* promoter (**Fig 1***b*). Furthermore, we found a consensus sequence for binding to the homeodomain (ATTAAT) and paired domain (GGAAC) between position –263 and –238. *In vitro* translated/transcribed PAX3 protein was found to bind the promoter sequence around this consensus sequence specifically as judged by EMSA (data not shown). Promoter constructs that do not contain this sequence were not activated in melanoma cells (**Fig 1***c*).

Next we examined the effect of *PAX3* mutations observed in WS1 on *MITF* transactivation using reporter assay and EMSA. We selected three PAX3 mutations that usually occur in the paired domain or homeodomain (**Fig 2**); Pro50Leu, Arg271Gly, Try266Cys. All of these PAX3 mutations failed to bind or transactivate the *MITF* promoter (**Fig 3**). These findings show that *MITF* is a target gene of *PAX3* and provide a molecular basis for the hypopigmentation and hearing impairment of individuals with WS1.

*Pax-3* deficient *splotch* mice (Bober *et al*, 1994) and *PAX3* deficient WS type 3 (WS3) individuals (Hoth *et al*, 1993) display limb muscle hypoplasia. *Pax-3* plays an important role in appropriate migration of myoblasts to the limb bud (Bober *et al*, 1994; Goulding *et al*, 1994; Williams and Ordahl, 1994). Thus, *PAX3/Pax-3* likely regulates genes involved in muscle differentiation. Product of an oncogene, *c-met*, is also required for limb muscle development (Bladt *et al*, 1995), and this gene encodes the receptor for hepatocyte growth factor of a receptor tyrosine kinase family (Bottaro *et al*, 1991). Epstein *et al* (1996) revealed that *c-met* is a target gene of *Pax-3*. First, they showed reduction of *c-met* expression in *splotch* mice. Next, they showed Pax3 can stimulate *c-met* expression in cultured cells. They found putative Pax3 DNA-binding sites in the human *c-MET* promoter. Muscle differentiation and development are dependent on myogenic genes, *MyoD*, *Myf5*, *MyoD*, and *myogenin*. Recently, it was shown that infection of embryonic tissue with a retrovirus encoding Pax-3 is sufficient to induce expression of *MyoD*, *Myf-5*, and *myogenin* (Maroto *et al*, 1997). Consistent with this finding, investigation of *splotch/Myf-5*$^{-/-}$ double mutant revealed that MyoD acts genetically downstream of *Pax-3* and *Myf-5* (Tajbakhsh *et al*, 1997).

Our previous finding of tyrosinase and TRP-1 induction in permanent *MITF* transfectants (Tachibana *et al*, 1996; Tachibana, 1997) implies that genes for these enzymes are downstream genes of *MITF*. To support this notion it was shown that promoters of the *tyrosinase* and *TRP-1* genes were transactivated by *MITF* through binding with an M-box in these promoters (for review see Shibahara *et al*, 1998). In contrast, the promoter of the *TRP-2* gene was not transactivated by *MITF* despite the existence of an M-box in the promoter (Yasumoto *et al*, 1997). Consistent with this finding, we were able to induce melanocytic characteristics by *MITF* in fibroblasts that constitutively express TRP-2 but not in other fibroblasts that do not express it (Tachibana *et al*, 1994). Others found that *TRP-2* expression did not depend on *mi* in retinal pigment cell (Opdecamp *et al*, 1997).

The *c-kit* promoter is transactivated by *mi*, and overexpression of *mi* rescues the low c-kit expression in mast cells lacking wild-type *mi* (Tsujimura *et al*, 1996). Mi upregulated c-Kit expression in melanocyte precursor cells of neural crest, although onset of *c-kit* was independent

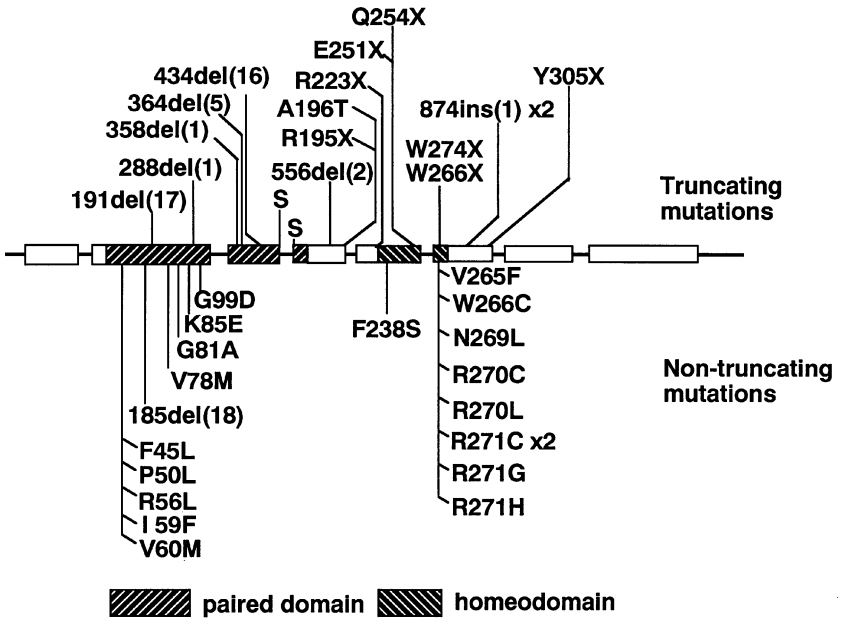


**Figure 2. Diagram of the *PAX3* gene showing location of mutations detected.** Each box indicates an exon. Numbers in parentheses denote the numbers of deleted nucleotides. Non-truncating mutations are expected to encode a full-length PAX3 with a substitution of an amino acid at the paired or homeodomain. S, splice-site mutation (modified from Tassabehji *et al*, 1995; mutations cited by Baldwin *et al*, 1995; Tassabehji *et al*, 1995; Watanabe *et al*, 1998 are shown).

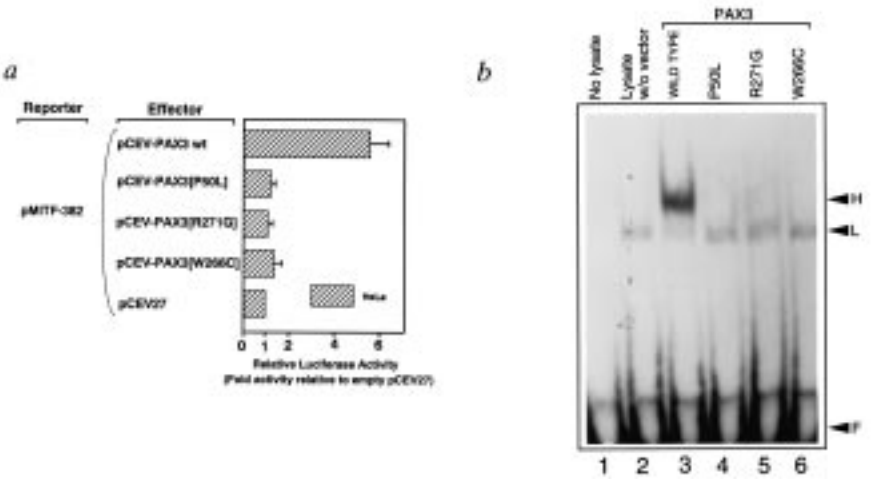


**Figure 3. Mutant PAX3 failed to bind and transactivate the *MITF* promoter.** (*a*) EMSA: PAX3[P50 L] (predicting the substitution of an amino acid in the paired domain) and PAX3[R271G]/PAX3[W266C] (predicting the substitution of an amino acid in the homeodomain) fail to bind the consensus sequence (ATTAATAC-TACTGGAAC) in the *MITF* promoter sequence, whereas wild-type PAX3 does bind the sequence. *Arrowheads* identify higher (H) and lower (L) DNA-protein complexes, and the free probe (F). The higher complex (H) is sequence specific and contains PAX3 protein, whereas the lower complex (L) is not sequence specific and does not contain PAX3 (not shown). (*b*) Reporter assay: Co-transfection of mutated *PAX*3 cDNA, designed from mutations found in WS1, failed to transactivate the *MITF* promoter, whereas wild-type *PAX*3 cDNA did transactivate. (Reproduced from Watanabe *et al*, 1997 with permission.)

of *mi* (Opdecamp *et al*, 1997). Stimulation of c-Kit receptor in melanoma cells activated MAP kinase, which in turn phosphorylated Mi by recruiting p300/CBP and consequently upregulated the tyrosinase gene (Hemesath *et al*, 1998; Price *et al*, 1998). These findings suggest that *c-kit* is a downstream gene of *mi* and interaction of Mi and that c-Kit is crucial for melanocyte development. Mutaions of *c-KIT* cause phenotypes similar to WS: piebaldism associated with deafness in human (Spitz and Beighton, 1998). Another gene shown to be downstream of *mi* is the quail *QNR-71* gene, which is expressed specifically in pigment layers and encodes a protein homologous to melanosomal matrix protein. The promoter of this gene is transactivated by two b-HLHZip proteins, i.e., Mi and c-MYC (Turque *et al*, 1996).

WS associated with Hirschprung disease, i.e., aganglionic megacolon, is classified as WS type 4 (WS4). Mutations of three genes have been identified in WS4: genes for endothelin-B receptor or endothelin 3 and the *SOX10* gene. Disruption of the murine endothelin-3 (*Edn3*) gene and the endothelin-receptor (*Ednrb*) gene results in aganglionic megacolon and spotted coat color in mice (Baynash *et al*, 1994; Hosoda *et al*, 1994). Human mutations of *EDN3* (Edrey *et al* 1996; Hofstra *et al*, 1996) and *EDNRB* (Puffenberger *et al*, 1994) are associated with WS4 families. Mutations of murine *Sox10* or human *SOX10*, encoding a transcription factor with the HMG DNA-binding motif, were found, respectively, in *Dom* mice with megacolon (Pingault *et al*, 1998) and WS4 families (Southard-Smith *et al*, 1998). Thus, interaction of EDN3/Edn3 and EDNRB/Ednrb, and activation of *SOX10*/*Sox10*, seem to be essential for development of epidermal melanocytes and enteric neurons; however, the question of how these gene products fit in the cascade of WS genes remains to be answered. While Edn3 enhanced the number of melanocytes in wild-type neural crest cultures (Reid *et al*, 1996), it did not do so in those from *mi*-mutated mice (Opdecamp *et al*, 1997). These findings are consistent with the notion that *Endrb* is the downstream of *mi*, and, consequently *Endrb* is not expressed in neural crest cells of *mi*-mutant mice. Because *Ednrb* positive cells are expressed in migrating neural crest cells of 10.5-d.p.c. $Sox10^{Dom}/Sox10^{Dom}$ embryos (Southard-Smith *et al*, 1998), *Ednrb* seems to be independent of Sox10. Furthermore, since *Edrnb*-and *TRP-1*-

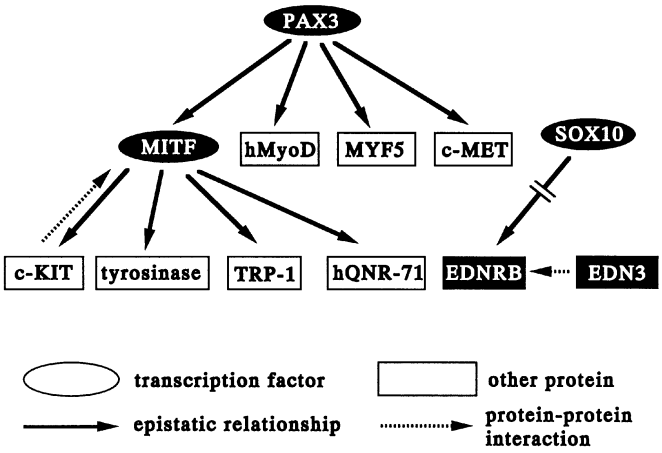


**Figure 4. A cascade of genes and/or their products related to Waardenburg syndrome.** Ovals represent transcription factors, and rectangles represent other types of proteins; those responsible for WS are in black. PAX3, human MyoD, MYF5, and c-MET are involved in myocyte differentiation/proliferation, whereas PAX3, MITF, SOX10, c-KIT, tyrosinase, TRP-1, human QNR-71, EDNRB, and EDN3 are involved in melanocyte differentiation/proliferation. It is unlikely that SOX10 regulates EDNRB, and thus the downstream genes of SOX10 remains to be elucidated.

positive cells were virtually absent in neural crest of the 11.5-d.p.c embryos (Southard-Smith *et al*, 1998), *Sox10* seems to determine the neural crest cell fate.

To summarize, three transcription factor genes, *PAX3*, *MITF*, and *SOX10*, have been shown to be responsible for WS. By epistatic relationship, the *PAX3* gene most likely regulates the human *MyoD*, *MYF5*, and *c-MET* genes, whereas the *MITF* gene regulates genes for c-KIT, tyrosinase, TRP-1, and human QNR-71. In addition, we determined the epistatic relationship of *PAX3* and *MITF* (Watanabe *et al*, 1998). Further studies will determine whether there is an epistatic relationship between *SOX10* and *MITF* or *PAX3*. Completion of cascade-like network among genes and/or gene products related to WS (**Fig 4**) will help us understand the mechanism of the manifestation of WS symptoms.

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